JOURNAL OF VIROLOGY, Feb. 2005, p. 2301–2308 Vol. 79, No. 4
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.4.2301–2308.2005

The Adenovirus E4-6/7 Protein Directs Nuclear Localization of E2F-4 via an Arginine-Rich Motif

Joel E. Schaley,† Marina Polonskaia, and Patrick Hearing*

Department of Molecular Genetics and Microbiology, School of Medicine, Stony Brook University, Stony Brook, New York

Received 22 July 2004/Accepted 5 October 2004

E2F transcription factors are key participants in the regulation of proliferation, apoptosis, and differentiation in mammalian cells. E2Fs are negatively regulated by members of the retinoblastoma protein (pRb) family. During adenovirus (Ad) infection, viral proteins that displace pRb family members from E2Fs and recruit E2F complexes to viral and cellular promoter regions are expressed. This recruitment of E2F involves the induction of stable E2F binding to inverted E2F binding sites in the Ad E2a and cellular E2F-1 promoters and induces both viral and cellular gene expression. E2F-4 has abundant E2F activity within cells, and the majority of E2F-4 in asynchronous cells is found in the cytoplasm. Upon expression of the adenovirus E4-6/7 protein, a significant portion of E2F-4 is translocated to the nucleus, and its activity constitutes the majority of Ad-induced nuclear E2F DNA binding activity. This redirection of E2F-4 from cytoplasm to the nucleus requires an N-terminal arginine-rich nuclear localization sequence within E4-6/7. The directed targeting of E4-6/7 to the nucleus is important for the function of this protein in the context of viral infection. This function of E4-6/7 has a redundant component as well as nonredundant components in cooperation with the adenovirus E1A oncoproteins to deregulate and usurp host cell E2F function.

E2F transcription factors are key players in the regulation of proliferation, apoptosis, and differentiation in mammalian cells (reviewed in references 26, 31, and 52). There are seven members of the E2F family. E2Fs form heterodimeric complexes with the DP family of proteins (DP-1 and DP-2). The E2F family may be divided into three subfamilies based on their binding to members of the retinoblastoma tumor suppressor family (pocket proteins retinoblastoma protein [pRb], p107, and p130) (reviewed in reference 51). E2F-1, E2F-2, and E2F-3 preferentially bind pRb, and E2F-4 and E2F-5 preferentially bind p107 and p130, while E2F-6 and E2F-7 lack C-terminal coding sequences required for pocket protein binding. The interaction of E2Fs with pRb family members occurs at specific stages of the cell cycle (51). In G_0, the predominant E2F complexes are E2F-4/DP and E2F-5/DP with p130. Following growth factor stimulation, the E2F-5/p130 complex diminishes, and E2F-1/DP bound to pRb and E2F-4/DP bound to p107 become evident. The activation of G_1 cyclin-dependent kinases (cyclin D/cdk4 and cyclin E/cdk2) results in hyperphosphorylation of pRb family members and their release from E2Fs. "Free" E2F activity is evident during late G_1 and into S phase of the cell cycle and transactivates promoters of E2F-responsive genes (26, 31, 52). E2Fs control the expression of genes involved in G_1 and S-phase progression. pRb family members interact with E2Fs via the conserved pocket domain, which mediates binding to DNA tumor virus oncoproteins (10) as well as sequences C-terminal of this region (51). The C-terminal regions of E2F-1 to E2F-5 also contain transcriptional activation sequences, although it is generally accepted that E2F-1 to E2F-3 play a major role in transcriptional activation of E2F-responsive genes, while E2F-4 and E2F-5 play a major role in the repression of these genes (see reference 53 and references therein; reviewed in references 26, 31, 51, and 52). Several studies have supported a role for E2F-4 in transcriptional activation of gene expression (6, 25, 43), and consistent with this idea, E2F-4 interacts with transcriptional activators TRRAP and GCN5 (25).

E2F-1 was the first family member cloned, and it has well-characterized E2F activity (26). The E2F-1 protein has the properties of both an oncogene and a tumor suppressor, depending on the context in which E2F-1 function is analyzed. E2F-1 expression is sufficient to drive quiescent cells to enter S phase of the cell cycle (22, 46, 49) and to cooperate with activated Ras to transform cells in culture and induce tumors in nude mice (21, 50, 57). E2F-1 overexpression promotes hyperplasia and can cooperate with activated Ras or inactivated p53 to promote tumor formation (44, 45). E2F-1 overexpression also prevents terminal differentiation of certain cell types (13). These properties appear to relate to transcriptional activation by E2F-1 and its ability to turn on the expression of genes that promote cell cycle progression (e.g., the c-*myc*, B-*myb*, cdc25A, and cyclin E genes) and S phase (e.g., the DNA polymerase delta, PCNA, cyclin A, and cdc2 genes). The analysis of E2F-1 knockout mice has shown that the protein also has properties of a tumor suppressor, since these mice are prone to develop tumors (9, 59). In this context, the role of E2F-1 as a transcriptional repressor when bound to pRb is revealed. Thus, E2F-1 plays roles in cell growth, death, and differentiation decisions.

E2F was first described as having nuclear activity and binding to an inverted binding site in the adenovirus (Ad) E2a promoter (23). The binding of E2F to these sites is stimulated by the Ad E4-6/7 protein, which induces the cooperative and

* Corresponding author. Mailing address: Stony Brook University, School of Medicine, Department of Molecular Genetics and Microbiology, Stony Brook, NY 11794-5222. Phone: (631) 632-8813. Fax: (631) 632-8891. E-mail: phearing@ms.cc.sunysb.edu.

† Present address: Department of Biology, Indiana University, Bloomington, Indiana.

stable binding of E2F to the E2a promoter via induction of E2F dimerization. The induction of E2F binding to the Ad E2a promoter in vitro directly correlates with transcriptional activation of the E2a promoter in vivo (19, 33, 35). The Ad E4-6/7 protein also transactivates the cellular E2F-1 promoter via the induction of E2F DNA binding to an inverted configuration of binding sites at the sites of initiation of E2F-1 transcription (47). These results suggest that the Ad E4-6/7 protein may function as an analogue to a cellular counterpart that regulates E2F dimerization and stable DNA binding. Indeed, p107 induces stable E2F-4/DP binding to inverted E2F binding sites in the Ad E2a and cellular E2F-1 promoters (38). Such induction of E2F DNA binding requires the pocket domain of p107. Induction of E2F DNA binding by p107 occurs primarily in S phase, when p107 protein levels increase significantly, and directly correlates with transcriptional repression. The related tumor suppressor, p130, also mediates this function (39). Finally, the E4-6/7 protein plays a redundant role with the E1A oncoproteins by binding to internal sequences within E2Fs (the marked box region) to competitively release pocket proteins from E2Fs (38). Under certain circumstances, E4-6/7 may replace some of the functions of E1A to complement the growth of an E1A-deficient adenovirus mutant (37).

Consistent with important roles in determining cell life, death, and differentiation, E2Fs are regulated by multiple mechanisms. Different members of the E2F family are regulated in the following ways: (i) negatively regulated by Rb family members (51); (ii) positively regulated at the level of transcription (2, 18, 21, 48); (iii) positively and negatively regulated by posttranslational phosphorylation, acetylation, and ubiquitin-dependent, proteasome-mediated degradation (5, 8, 15, 17, 24, 28–30, 58); and (iv) regulated through changes in subcellular localization (3, 27, 32, 55). Relative to the latter level of regulation, E2F-1, E2F-2, and E2F-3 contain nuclear localization sequences (NLSs) that direct nuclear targeting. In contrast, E2F-4 and E2F-5 lack NLSs and rely on binding partners, such as p107 and p130, which themselves contain NLSs, to carry these factors to the nucleus (3, 27, 32, 55). Further, E2F-4 contains two nuclear export sequences that direct CRM1-dependent cytoplasmic transport in the absence of pocket protein binding (11). E2F-4 nuclear localization is regulated during the cell cycle to optimize nuclear accumulation of E2F-4/p107 heteromers during S phase when repression of E2F target genes is initiated (55). Since the adenovirus E1A proteins disrupt E2F-4/p107 complexes (10), it would be anticipated that E1A could disallow E2F-4 nuclear localization during adenovirus infection and effect E2F-4 cytoplasmic accumulation. In this report, we demonstrate that the adenovirus E4-6/7 protein contains an arginine-rich nuclear localization signal that directs E2F-4 to the nucleus. Nuclear E2F-4 bound with E4-6/7 binds to the Ad early region 2 promoter, consistent with previous reports that E2F-4 transactivates this viral early promoter region.

MATERIALS AND METHODS

Cells, viruses, and plasmids. American Type Culture Collection HeLa and 293 cells were maintained in Dulbecco's modified minimal essential medium containing 10% calf serum. The virus stocks Ad5 WT300, dl356, dl356-CMV-E4-6/7-WT, and dl356-CMV were propagated in 293 cells and were described previously (14, 37). Mutant dl356 carries a 2-base-pair frameshift mutation in E4-ORF7 coding sequences (14) that eliminates the ability of the E4-6/7 protein to

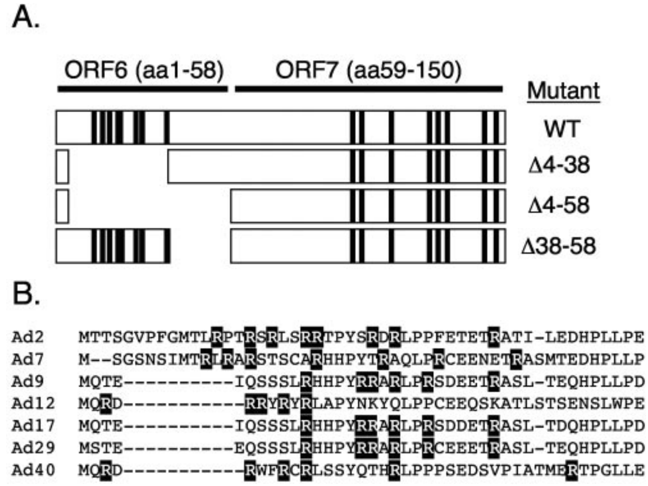

FIG. 1. E4-6/7 protein. (A) The wild-type E4-6/7 protein is translated from a spliced mRNA corresponding to amino acids 1 to 58 of E4-ORF6 fused to E4-ORF7 (amino acids 59 to 150). The wild-type protein (150 amino acids) and in-frame deletion mutant derivatives (Δ4–38, Δ4–58, and Δ38–58) are depicted. Vertical bars indicate arginine residues. (B) Sequence alignment of predicted N-terminal E4-6/7 coding sequences from adenoviruses of indicated serotypes. The sequence alignment was performed with the ClustalW Multiple Sequence Alignment program of the Baylor College of Medicine Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi/align/multi-align.pl). Arginine residues are highlighted with black boxes.

interact with E2Fs (36). dl356-CMV contains the dl356 deletion as well as a deletion within the E1A region (nucleotides 355 to 811) and an insertion of the cytomegalovirus (CMV) promoter-enhancer at the E1 deletion junction. dl356-CMV-E4-6/7-WT additionally carries a wild-type E4-6/7 cDNA downstream of the CMV promoter-enhancer; E1B splicing and polyadenylation signals are utilized to direct processing of RNAs derived from the E4-6/7 cDNA (36). Viral mutants dl356-CMV-E4-6/7-Δ4-58, dl356-CMV-E4-6/7-Δ4-38, and dl356-CMV-E4-6/7-Δ38-58 are isogenic with dl356-CMV-E4-6/7-WT but carry in-frame deletions in E4-6/7, as described previously (36) and shown in Fig. 1A. Purified virus particles were prepared by using CsCl equilibrium gradient centrifugation. Cells were infected with 200 virus particles/cell for 1 h at 37°C, the virus inoculum was removed, and fresh medium was added.

Plasmids that express wild-type and mutant E4-6/7 proteins were previously described (36). An expression vector containing enhanced green fluorescent protein (EGFP) (pEGFP-C1; Clontech) fused at the N terminus of full-length, wild-type E2F-4 was generated by standard cloning approaches. The N-terminal 58 amino acids of E4-6/7 protein were synthesized by PCR and fused at the N terminus of EGFP in vector pEGFP-N1 (Clontech). A mutant that changes six arginine residues to alanine residues (residues 16, 18, 21, 22, 27, and 29) was generated in this background by Quick-Change mutagenesis (Stratagene) (Fig. 1B).

Metabolic labeling, cell fractionation, immunoprecipitation, and Western blot analysis. For ^{35}S pulse-labeling, HeLa cells were infected as described above. Six hours postinfection, the cells were washed and incubated in methionine/cysteine-free medium containing 2% calf serum for 30 min. Cells were pulse-labeled by using 1 mCi/100-mm-diamater dish of ^{35}S-Met-^{35}S-Cys in methionine/cysteine-free medium containing 2% calf serum for 1 to 2 h at 37°C and then harvested. Nuclear and cytoplasmic fractions for subcellular localization analyses were prepared as described previously (56). Briefly, cells were washed with phosphate-buffered saline (PBS) and resuspended in hypotonic detergent buffer (0.3 M sucrose, 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.4% NP-40, 0.5 mM dithiothreitol). The cells were gently vortexed, and nuclei were precipitated at 1,000 $\times$ g for 5 min. Pelleted nuclei were washed with hypotonic buffer containing detergent, repelleted, and resuspended in nuclear extraction buffer (5). Cytoplasmic fractions were adjusted to 140 mM NaCl, and all extracts were centrifuged at 100,000 $\times$ g for 20 min.

For E4-6/7 immunoprecipitation, nuclear and cytoplasmic proteins from the equivalent of 1 $\times$ 10^7 cells were brought to 0.1 M Tris, pH 8.0, 2% sodium dodecyl sulfate (SDS), and 5 mM β-mercaptoethanol and boiled for 5 min. Samples were diluted into 20 volumes of RIPA buffer (160 mM NaCl, 10 mM

Tris, pH 7.4, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS). Each fraction was precleared for 2 h with protein A-Sepharose beads. Fifty microliters of anti-E4-ORF7 monoclonal antibody M80 (35) was added, and immunoprecipitation was carried out overnight on ice. Antibody was captured by using protein A-Sepharose beads washed with RIPA buffer, high-salt buffer (1 M NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40), and RIPA buffer. Immune complexes were boiled in Laemmli sample buffer and resolved by using SDS–15% polyacrylamide gel electrophoresis (PAGE). Gels were fixed, treated with NAMP 100 (Amersham), dried, and exposed to X-ray film.

For Western blot analysis, 20 to 60 μg of total protein was resolved by SDS-PAGE, and proteins were transferred to a nitrocellulose or Nylon P membrane (Amersham) and probed by using primary and secondary antibodies for enhanced chemiluminescence (Amersham) according to the manufacturer's instructions. E4-6/7 proteins were detected by using M80 (35); E2F-4 was detected by using monoclonal antibody Ab-4 4E2F04 (LabVision). The purity of nuclear and cytoplasmic fractions was verified by Western blot analysis using a monoclonal antibody against p53 (pAB240; sc-99; Santa Cruz Biotechnology) and a polyclonal antibody against paxillin (sc-7336; Santa Cruz Biotechnology).

Transfection assays and immunofluorescence. American Type Culture Collection HeLa cells plated on glass coverslips were transfected overnight with different plasmids with Fugene 6 (Roche), as recommended by the manufacturer. Following transfection, cells were washed and processed by fluorescence microscopy or infected with the viruses indicated. For microscopy, cells were washed with PBS solution and fixed in 3% formaldehyde for 1 h. Fixed cells were washed with PBS and blocked with PBS containing 10% goat serum. E4-6/7 proteins were detected by using monoclonal antibodies M45 or M80 (35) or a rabbit polyclonal anti-E4-6/7 antiserum (38). The secondary antibody was tetramethyl rhodamine isocyanate (TRITC)-goat anti-mouse immunoglobulin G (Zymed). EGFP and TRITC signals were visualized and photographed on a Zeiss Axiovert 135 microscope.

Extract preparation and gel mobility shift assays. All buffers were used at 4°C and contained a cocktail of protease inhibitors. Nuclear extracts for gel mobility shift assays were prepared according to the method of Dignam et al. (7) and dialyzed against DB-100 (20 mM HEPES, pH 7.5, 100 mM KCl, 10% glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). In vitro DNA binding assays were performed as described previously (38). Briefly, binding reaction mixtures (20 μl) contained 5 to 10 μg of nuclear extract, 2 μg of sonicated salmon sperm DNA, and 20,000-cpm ^{32}P-labeled Ad E2a E2F recognition sites (1 to 2 fmol of DNA) in DB supplemented with Nonidet P-40 (final concentration, 0.1%). Binding reactions were incubated for 1 to 2 h at room temperature, followed by electrophoresis on a 4% 30:1 polyacrylamide gel run in 0.5× Tris-borate-EDTA at 4°C. The Ad E2a E2F probe contains nucleotides −30 to −73 from the Ad5 E2a promoter (38).

RESULTS

The N-terminal 58 amino acids of E4-6/7 are sufficient to confer nuclear localization. The N-terminal 58 amino acids are shared by the E4-ORF6 and E4-6/7 proteins (Fig. 1). The C-terminal 92 amino acids of E4-6/7 are necessary and sufficient to bind E2Fs and induce DNA binding to viral and cellular promoter regions (36). Thus far, no particular function has been ascribed to the N-terminal domain of E4-6/7. Three mutants with mutations in the N-terminal region of E4-6/7 were constructed, containing in-frame deletions of amino acids 4 to 38, 4 to 58, and 38 to 58 (Fig. 1). Plasmid vectors directing the expression of wild-type and mutant E4-6/7 proteins were transfected into HeLa cells, and E4-6/7 localization was assessed by indirect immunofluorescence by using a rabbit polyclonal anti-E4-6/7 antibody that recognizes all four proteins (Fig. 2). The signal of wild-type E4-6/7 was entirely nuclear, indicating that no other Ad proteins were required for this to occur. The mutant protein lacking amino acids 4 to 58 (the E4-ORF6 segment) was equally partitioned between the nucleus and cytoplasm. The diffuse localization of this mutant implies that E2F binding is not responsible for nuclear accumulation, since this protein binds E2Fs as efficiently as wild-type E4-6/7 (36). Analysis of the localization of the other two E4-6/7 mutants indicates that amino acids 4 to 38 are required for nuclear accumulation, whereas amino acids 38 to 58 are dispensable for this function. Thus, E4-6/7 appears to contain a nuclear targeting signal within the N-terminal region of the protein. These results, and those presented below, were consistently observed in cells that were examined in numerous different experiments and with numerous fields of cells within each experiment.

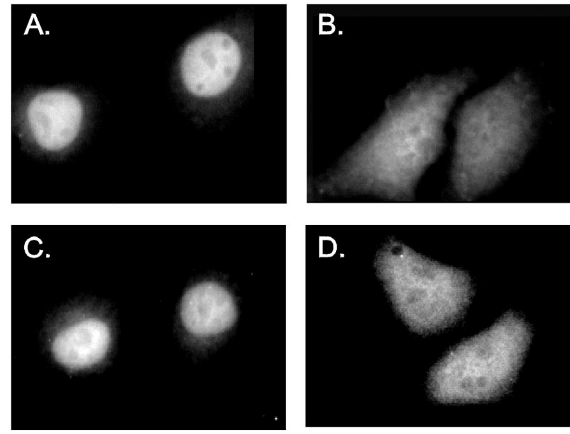

FIG. 2. Localization of wild-type and mutant E4-6/7 proteins. HeLa cells were transfected with expression vectors for the wild-type and mutant E4-6/7 proteins. Cells were fixed, and E4-6/7 proteins were visualized by using indirect immunofluorescence with a rabbit polyclonal antibody directed against the full-length E4-6/7 protein. Wild-type E4-6/7 (A), E4-6/7-Δ4–58 (B), E4-6/7-Δ38–58 (C), and E4-6/7-Δ4–38 (D) are shown. Proteins were visualized by using a fluorescein isothiocyanate (FITC) filter on a fluorescence microscope.

To confirm these findings, HeLa cells were infected with Ad5 derivatives of dl356 (an E4-ORF7 truncation mutant) containing CMV-driven expression cassettes for each E4-6/7 product in place of the E1A region. Six hours after infection, cells were pulse-labeled with radioactive methionine/cysteine, and nuclear and cytoplasmic fractions were prepared. Wild-type and mutant E4-6/7 products were immunoprecipitated with a monoclonal antibody (M80) directed against a C-terminal epitope common to all four proteins that does not recognize the truncated E4-6/7 product produced by mutant dl356 (35). Immune complexes were analyzed by SDS-PAGE, and proteins were visualized by autoradiography (Fig. 3). The sizes and localization patterns of these proteins were consistent with the immunostaining results. The wild-type and Δ38–58 proteins were predominantly nuclear, whereas the Δ4–58 and Δ4–38 mutant proteins were found in both nuclear and cytoplasmic fractions, although the Δ4–38 protein was slightly, but reproducibly, enriched in the nuclear fraction. Since the 19-kDa E4-6/7 protein is small enough to freely diffuse through the nuclear pore complex (1), we conclude from these results that the N-terminal region of E4-6/7 is required for nuclear accumulation.

E4-6/7 contains an arginine-rich nuclear localization signal. We tested whether the N-terminal region of E4-6/7 was sufficient to direct nuclear targeting by fusing E4-ORF6 amino acids 1 to 58 to the N terminus of EGFP. The EGFP molecule is small enough to freely diffuse into the nucleus (1), and nuclear and cytoplasmic accumulation of EGFP was observed in transfected HeLa cells (Fig. 4). In contrast, fusion of E4-ORF6 amino acids 1 to 58 to EGFP resulted in complete nuclear localization, indicating that this region carries an autonomous

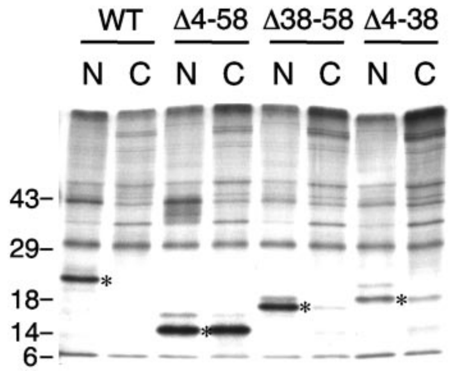

FIG. 3. Subcellular localization of wild-type and mutant E4-6/7 proteins. HeLa cells were infected with E1A-negative viruses in a dl356 background (see Materials and Methods) that express wild-type and mutant E4-6/7 proteins. WT, dl356-CMV-E4-6/7-WT; Δ4–58, dl356-CMV-E4-6/7-Δ4–58; Δ38–58, dl356-CMV-E4-6/7-Δ38–58; and Δ4–38, dl356-CMV-E4-6/7-Δ4–38. Six hours after infection, cells were radiolabeled with ^{35}S-methionine/cysteine and fractionated into nuclear (N) and cytoplasmic (C) fractions, as described in Materials and Methods. E4-6/7 proteins were immunoprecipitated by using a monoclonal antibody (M80) directed against the C terminus of E4-ORF7. Protein expression was analyzed by SDS-PAGE and autoradiography. Wild-type and truncated E4-6/7 proteins are indicated by asterisks; molecular weight standards are indicated on the left.

NLS. A comparison of the equivalent E4-ORF6 amino acid sequences from a variety of evolutionarily diverse human adenoviruses is depicted in Fig. 1B. The sequences of the commonly studied serotypes Ad2 and Ad5 are identical in this region. The other serotypes show significant homology both to the Ad2 and Ad5 sequences and to each other. The presence of an arginine-rich region in the C terminus of E4-ORF6 has already been identified as directing nuclear localization and retention of the E4-ORF6 protein (40, 41). There are eight arginine residues located between amino acids 13 and 38 of Ad2 and Ad5 E4-ORF6, which equates with greater than 25% arginine content for this region. The overall arginine content of other Ad5 E4 proteins is 4 to 5%, suggesting a clustering of arginines in the N-terminal segment of E4-ORF6. While the position of specific arginine residues is not conserved per se in this region in other Ad serotypes, the overall clustering of arginine residues in the N terminus of E4-ORF6 is maintained. A mutant that changed arginine residues 16, 18, 21, 22, 27, and 29 to alanines in the context of the fusion protein of EGFP and E4-ORF6 amino acids 1 to 58 was made. These mutations eliminated nuclear localization of the fusion protein, demonstrating that the N terminus of E4-6/7 contains an arginine-rich NLS.

E4-6/7 directs nuclear localization of E2F-4. The E4-6/7-induced E2F complex has been reconstituted from partially purified components (38). The addition of E2F-1, DP-1, and E4-6/7 to a binding reaction with an Ad E2a promoter probe fragment yielded a complex with mobility and characteristics identical to that observed with Ad5-infected cell nuclear extract. E4-6/7 has been shown by this approach to interact with five of the seven known E2F species (38). Addition of a monoclonal antibody (M45) that recognizes an N-terminal epitope in E4-6/7 supershifted all Ad-induced E2F complexes observed with an E2a probe in a mobility shift assay (Fig. 5A), whereas an unrelated monoclonal antibody was without effect. A specific antibody against E2F-4 supershifted a large percentage of the Ad-induced E2F complex in this assay, a result that was somewhat surprising given the prediction that the release of pocket proteins from E2F-4 by the E1A products would allow the nuclear export sequences of E2F-4 to direct this protein from the nucleus. These results suggested that E4-6/7 may confer nuclear localization to E2F-4. Consistent with this idea, infection of cells with a virus that expresses a mutated E4-6/7 protein that lacks the N-terminal region (mutant Δ4–58) did not result in significant induction of nuclear E2F DNA binding activity (Fig. 5B) despite the fact that this mutant form of E4-6/7 is perfectly capable of inducing E2F binding to the Ad E2a promoter when expressed and analyzed in vitro (36).

Previous results have shown that E2F-4 expressed alone by transfection assay is found predominantly in the cytoplasm (3, 27, 55). An identical result was found in transfected HeLa cells by using an EGFP–E2F-4 fusion protein (Fig. 6). To test whether E4-6/7 expression directs E2F-4 into the nucleus, cells transfected with the EGFP–E2F-4 expression vector were infected with wild-type and recombinant Ad5 (Fig. 6). Infection with wild-type Ad5 resulted in nuclear E2F-4 in a pattern colocalizing with the E4-6/7 product. This effect could be attributed to many viral products. To specifically test the role of E4-6/7 in E2F-4 nuclear localization, two assays were performed. First, cells were infected with dl356, an otherwise wild-type Ad5 virus that expresses a truncated E4-6/7 protein that is incapable of binding E2Fs (36). While the truncated E4-6/7 protein was found to be nuclear, as expected from results described above, E2F-4 displayed a pattern of cytoplasmic localization that was indistinguishable from transfected but uninfected cells. Second, transfected cells were infected with the E1-negative recombinant virus that expresses the wild-type E4-6/7 protein but not other viral products in the context of short-term assays (38). Expression of the wild-type E4-6/7 protein also resulted in nuclear E2F-4 accumulation in a pattern coincident with E4-6/7.

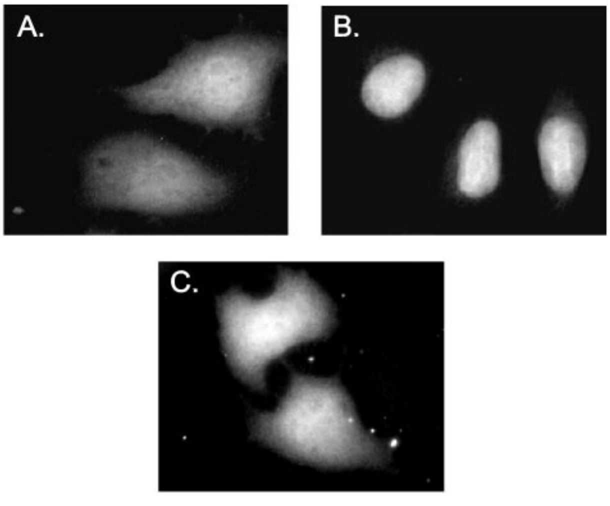

FIG. 4. The N-terminal region of E4-6/7 contains an NLS. HeLa cells were transfected with expression plasmids for EGFP (A), EGFP fused to the N-terminal 58 amino acids of E4-6/7 (B), and E4-6/7 amino acids 1 to 58 containing six arginine-to-alanine substitution mutations (residues 16, 18, 21, 22, 27, and 29) (Fig. 1B) (C). EGFP proteins were visualized by using an FITC filter on a fluorescence microscope.

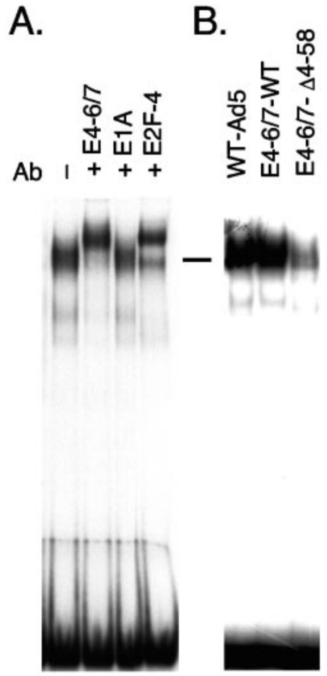

FIG. 5. Ad-induced E2F complexes in infected cell nuclear extracts. (A) Nuclear extract from wild-type Ad5-infected HeLa cells was used in gel mobility shift assays with an Ad E2a E2F site promoter region probe. Antibodies directed against Ad E4-6/7, Ad E1A, or E2F-4 were added following the binding reaction and prior to electrophoresis. DNA-protein complexes supershifted by antibody are evident by reduced mobility in the gel. (B) Nuclear extract was isolated from HeLa cells infected with wild-type Ad5 (WT-Ad5), dl356-CMV-E4-6/7-WT (E4-6/7-WT), or dl356-CMV-6/7-Δ4–58 (E4-6/7-Δ4–58) viruses. Gel mobility shift assays were performed as described for panel A. Ad-induced E2F complexes are indicated by a horizontal line; unbound probe DNA is evident at the bottom of the gels.

To confirm these findings, cell fractionation experiments were performed. HeLa cells were infected with wild-type Ad5 and recombinant viruses. Nuclear and cytoplasmic fractions were prepared and immunoblotted for endogenous E2F-4 levels (Fig. 7). The specificity of subcellular fractionation was assessed by immunoblot analysis using antibodies against p53 (nuclear) and paxillin (cytoplasmic). With uninfected cells, the vast majority of E2F-4 was cytoplasmic. Following infection with wild-type Ad5, nuclear E2F-4 was observed, although a significant amount remained cytoplasmic. Infection with recombinant virus vectors provided results consistent with those presented above. Expression of wild-type and Δ38–58 E4-6/7 resulted in nuclear E2F-4, whereas expression of Δ4–58 and Δ4–38 E4-6/7 did not. Expression of E1A without the E4-6/7 protein did not alter E2F-4 localization. We conclude from these results that the E4-6/7 protein relocalizes E2F-4 to the nucleus in a manner that is dependent on the E4-6/7 nuclear localization signal. Unlike all previously characterized functions of E4-6/7, redirection of E2F-4 to the nucleus requires both the E4-ORF6 amino acids 1 to 58 and E4-ORF7 modules.

The N terminus of E4-6/7 contributes to viral growth efficiency. We previously reported that E4-6/7 protein expression partially complements the loss of E1A in the context of infection with recombinant viruses (37), reiterating the notion that there is some functional redundancy between the E1A and the E4-6/7 products. HeLa cells were infected with wild-type Ad5 or E1-negative viruses that express wild-type E4-6/7, E4-6/7-Δ4–58, or no E4-6/7 product, and viral growth kinetics was measured. The lack of E1A and E4-6/7 expression reduced virus growth more than 5 orders of magnitude. Expression of wild-type E4-6/7 in the absence of E1A augmented virus growth more than 1,000-fold. In this context, deletion of the N-terminal region of E4-6/7 that contains the nuclear localization signal reduced virus growth more than 50-fold. Results shown in Fig. 3 as well as immunoblot analysis (data not shown) demonstrated that the E4-6/7-Δ4–58 deletion mutant protein accumulated to levels that were equivalent to the wild-type E4-6/7 product. Thus, the directed targeting of E4-6/7 to the nucleus is important for the function of this protein in the context of viral infection.

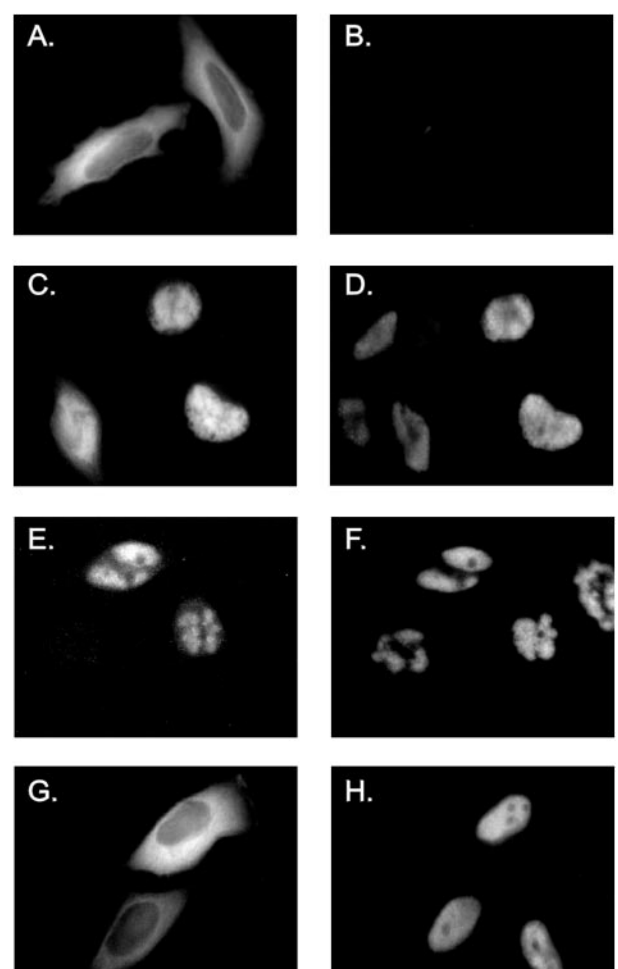

FIG. 6. Regulation of exogenously expressed E2F-4 localization by E4-6/7. HeLa cells were transfected with an expression vector for E2F-4 fused to EGFP. Transfected cells were left uninfected (A and B), were infected with wild-type Ad5 (C and D), or were infected with dl356-CMV-E4-6/7-WT (E and F) or dl356 (G and H). Cells were fixed and visualized for EGFP (A, C, E, and G) by using an FITC filter or E4-6/7 proteins (B, D, F, and H) by indirect immunofluorescence using monoclonal antibody M45 and TRITC-labeled secondary antibody.

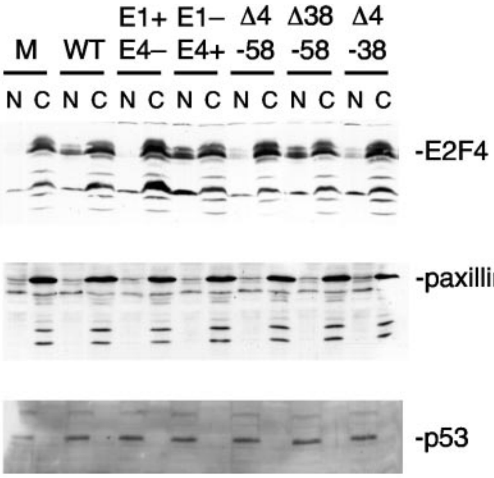

FIG. 7. Subcellular localization of endogenous E2F-4 is regulated by E4-6/7. HeLa cells were infected with wild-type Ad5 and dl356, E1A-negative derivatives that express wild-type and mutant E4-6/7 proteins. At 16 h after infection, nuclear and cytoplasmic fractions were prepared. Immunoblot analysis was used to determine the localization of E2F-4. p53 and paxillin were used as controls for the purity of nuclear and cytoplasmic fractions, respectively. M, mock-infected cells; WT, wild-type Ad5; E1+/E4−. dl356; E1−/E4+, Δ4–58, Δ38–58, and Δ4–38 are dl356-CMV viruses that express wild-type and mutant E4-6/7 proteins Δ4–58, Δ38–58, and Δ4–38, respectively.

DISCUSSION

The adenovirus E4-6/7 protein performs multiple functions within infected cells. First, the E4-6/7 protein interacts directly with E2F family members and mediates the cooperative and stable binding of E2Fs to a unique pair of inverted E2F binding sites in the Ad E2a promoter and cellular E2F-1 promoter (19, 47). With both promoters, the induction of E2F DNA binding directly correlates with transcriptional activation (33, 35, 36). Second, the E4-6/7 protein serves a redundant role with the Ad E1A oncoproteins to displace pRb family members from E2Fs (38). E1A accomplishes this role by binding to pocket proteins in regions that mediate E2F interaction (10). In a mechanistically distinct manner, E4-6/7 mediates pocket protein displacement from E2Fs by binding regions of E2F/DP heterodimers required for pocket protein interaction (38). In both cases, competitive displacement of pRb family members from E2F/DP family members takes place, and the result is deregulation of free E2F activity that may target the activation of cellular genes that promote S-phase progression. In this report, we describe an additional level of E2F regulation by E4-6/7 via nuclear translocation of cytoplasmic E2F-4. Published reports (3, 27, 55) and our own results (Fig. 7) indicate that the vast majority of E2F-4 in asynchronous cells is found in the cytoplasm. Upon expression of E4-6/7 protein, a significant portion of E2F-4 is translocated to the nucleus (Fig. 7) and constitutes the majority of Ad-induced nuclear E2F DNA binding activity (Fig. 5). This redirection of E2F-4 from cytoplasm to the nucleus by E4-6/7 requires an N-terminal arginine-rich nuclear localization sequence. Such a function was also reported for the pocket proteins that are required to translocate E2F-4 into the nucleus during normal cell cycle progression (3, 27, 55).

Further similarities exist between the function of the pocket proteins and that of Ad E4-6/7. We previously reported that p107 and p130 mediate the stable binding of E2Fs to inverted binding sites in the Ad E2a and cellular E2F-1 promoter regions in a manner analogous to that directed by E4-6/7 (39). The Ad E4-6/7 protein contains two separate C-terminal segments that are required for stable E2F binding in solution (35). A similar result was found with p107 binding to E2F-4, where both the A and B segments of the pocket domain are involved in protein-protein interaction (51). The Ad E4-6/7 protein provides an interface to mediate E2F dimerization on inverted E2F binding sites (36), and p107 and p130 perform a similar function (39). We propose that E4-6/7 evolved as an analogue to cellular pocket proteins but with a different purpose. Pocket proteins interact with the marked box regions and C-terminal sequences of E2Fs to mask the transactivation domain as well as recruit histone deacetylases to repress transcription of promoter regions (51). In contrast, E4-6/7 targets only the E2F marked box regions to reveal the C-terminal transctivation domains and allow transcriptional activation of promoter regions. Thus, the redundancy of E4-6/7 with E1A for pocket protein displacement from E2Fs serves the additional purposes of promoter activation and regulation of E2F subcellular distribution.

The N-terminal 58 amino acids contain a nuclear localization signal that is necessary to translocate and retain E4-6/7 and E2F-4 in the nucleus (Fig. 2, 3, 6, and 7). This represents a unique function of E4-6/7 that is not redundant with E1A products, whose activities effectively dissociate E2F/DP heterodimers from pocket proteins. This region also is sufficient to direct nuclear accumulation when fused to a heterologous protein (EGFP) (Fig. 4). This N-terminal domain in E4-6/7 is important for the proper function of this protein during viral infection (Fig. 8), and we speculate that this property reflects redirection of the abundant E2F-4 activity into the nucleus to activate transcription of the viral E2a promoter region.

The "classical" nuclear localization sequence found in many

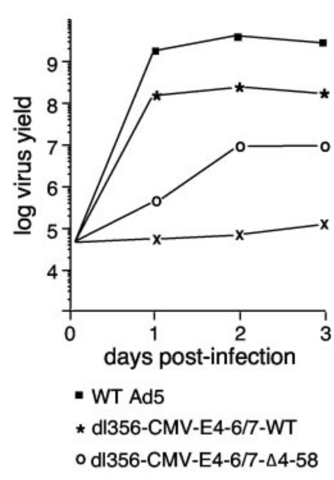

FIG. 8. Growth of E1-negative viruses that express wild-type and mutant E4-6/7 proteins. HeLa cells were infected with wild-type Ad5, E1A-negative viruses in a dl356 background that express wild-type E4-6/7 (dl356-CMV-E4-6/7-WT), E4-6/7 mutant Δ4–58 (dl356-CMV-E4-6/7-Δ4–58), or no E4-6/7 protein (dl356-CMV). Cellular lysates were prepared 4 h and 1, 2, and 3 days after infection, and infectious virus yield was determined by plaque assay of complementing 293 cells. The results represent the average of three independent experiments.

proteins contains monopartite or bipartite lysine-rich sequences (1). These sequences recruit importin α proteins involved in nuclear translocation. Importin α proteins in turn bind importin β proteins that effect nuclear translocation via the nuclear pore complex. A limited but growing list of proteins is translocated to the nucleus in the absence of importin α binding signals and subunits through direct interaction with importin β proteins. Cellular and viral proteins have been found to interact with importin β proteins via arginine-rich motifs; examples include human ribosomal proteins and human immunodeficiency virus (HIV) Tat and Rev (16, 20, 42, 54, 60). We believe that it is likely that E4-6/7 functions in an analogous manner. It is interesting to consider whether this region in E4-ORF6 plays a role in the nuclear translocation or nuclear retention of E4-ORF6. Recent reports described an arginine-rich amphipathic helix within the C terminus of E4-ORF6 that functions as a nuclear localization and/or nuclear retention signal (40, 41). The N-terminal arginine-rich sequence in E4-ORF6 also may play a role to direct nuclear transport of this protein, since E4-ORF6 mutant proteins that lack this region show both cytoplasmic and nuclear distribution, in contrast to wild-type E4-ORF6, where nuclear localization is observed (34).

It is generally accepted that E2F-1 to E2F-3 play a major role in transcriptional activation of E2F-responsive genes, while E2F-4 and E2F-5 play a major role in the repression of these genes (see reference 53 and references therein; reviewed in references 26, 31, 51, and 52). Our results are most consistent with the idea that adenovirus utilizes E2F-4 for transcriptional activation of the viral E2a promoter, and perhaps cellular promoters, via nuclear translocation and the induction of DNA binding by the E4-6/7 protein. Several studies have supported a role for E2F-4 in transcriptional activation of gene expression (6, 25, 43) and, consistent with this idea, E2F-4 interacts with transcriptional activators TRRAP and GCN5 (25). Interestingly, E2F-4 was found to stimulate production infection by bovine herpesvirus-1 (12), and HIV-1 Tat interacts with E2F-4 to stimulate the activity of E2F-dependent promoters, including the HIV long terminal repeat (4). These viruses may reveal transcriptional activation properties of E2F-4 not suggested in studies with cellular promoter regions, or perhaps these viruses have converted E2F-4 from a transcriptional repressor into a transcriptional activator in the context of viral infection.

In conclusion, adenovirus has transduced a number of cellular mechanisms for protein-protein interactions and regulation of E2F family members. Ad E4-6/7 and cellular pocket proteins interact with similar regions in E2Fs and modulate E2F-4 nuclear translocation. Ad E4-6/7 and pocket proteins also induce E2F binding to specific configurations of inverted E2F binding sites. We believe that the Ad E4-6/7 protein is a cellular analogue to the p107 and p130 tumor suppressor proteins. However, E4-6/7 function has been adapted for transcriptional activation instead of repression. This function of E4-6/7 has a redundant as well as an additive effect with the Ad E1A oncoproteins to deregulate and usurp E2F function. This effect stimulates S-phase progression in infected cells and achieves optimal transactivation of viral and cellular promoter regions.

ACKNOWLEDGMENTS

We thank our colleagues for many helpful discussions and Jihong Yang for excellent technical assistance.

This research was supported by Public Health Service grant CA28146 from the National Institutes of Health to P.H. J.E.S. was supported by NIH training grant CA09176.

REFERENCES

1. **Adam, S. A.** 1999. Transport pathways of macromolecules between the nucleus and the cytoplasm. Curr. Opin. Cell Biol. **11:**402–406.
2. **Adams, M. R., R. Sears, F. Nuckolls, G. Leone, and J. R. Nevins.** 2000. Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. Mol. Cell. Biol. **20:**3633–3639.
3. **Allen, K. E., S. de la Luna, R. M. Kerkhoven, R. Bernards, and N. B. La Thangue.** 1997. Distinct mechanisms of nuclear accumulation regulate the functional consequence of E2F transcription factors. J. Cell Sci. **110:**2819–2831.
4. **Ambrosino, C., C. Palmieri, A. Puca, F. Trimboli, M. Schiavone, F. Olimpico, M. Ruocco, F. di Leva, M. Toriello, I. Quinto, S. Venuta, and G. Scala.** 2002. Physical and functional interaction of HIV-1 Tat with E2F-4, a transcriptional regulator of mammalian cell cycle. J. Biol. Chem. **277:**31448–31458.
5. **Campanero, M. R., and E. K. Flemington.** 1997. Regulation of E2F through ubiquitin-proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. Proc. Natl. Acad. Sci. USA **94:**2221–2226.
6. **Choubey, D., and J. Gutterman.** 1997. Inhibition of E2F-4/DP-1 stimulated transcription by p202. Oncogene **15:**291–301.
7. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
8. **Dynlacht, B. D., O. Flores, J. A. Lees, and E. Harlow.** 1994. Differential regulation of E2F transactivation by cyclin/cdk2 complexes. Genes Dev. **8:**1772–1786.
9. **Field, S. J., F. Y. Tsai, F. Kuo, A. M. Zubiaga, W. G. Kaelin, Jr., D. M. Livingston, S. H. Orkin, and M. E. Greenberg.** 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell **85:**549–561.
10. **Frisch, S. M., and J. S. Mymryk.** 2002. Adenovirus-5 E1A: paradox and paradigm. Nat. Rev. Mol. Cell. Biol. **3:**441–452.
11. **Gaubatz, S., J. A. Lees, G. J. Lindeman, and D. M. Livingston.** 2001. E2F4 is exported from the nucleus in a CRM1-dependent manner. Mol. Cell. Biol. **21:**1384–1392.
12. **Geiser, V., and C. Jones.** 2003. Stimulation of bovine herpesvirus-1 productive infection by the adenovirus E1A gene and a cell cycle regulatory gene, E2F-4. J. Gen. Virol. **84:**929–938.
13. **Guy, C. T., W. Zhou, S. Kaufman, and M. O. Robinson.** 1996. E2F-1 blocks terminal differentiation and causes proliferation in transgenic megakaryocytes. Mol. Cell. Biol. **16:**685–693.
14. **Halbert, D., J. Cutt, and T. Shenk.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. **56:**250–257.
15. **Hateboer, G., R. M. Kerkhoven, A. Shvarts, R. Bernards, and R. L. Beijersbergen.** 1996. Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. Genes Dev. **10:**2960–2970.
16. **Hibbard, M. K., and R. M. Sandri-Goldin.** 1995. Arginine-rich regions succeeding the nuclear localization region of the herpes simplex virus type 1 regulatory protein ICP27 are required for efficient nuclear localization and late gene expression. J. Virol. **69:**4656–4667.
17. **Hofmann, F., F. Martelli, D. M. Livingston, and Z. Wang.** 1996. The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. Genes Dev. **10:**2949–2959.
18. **Hsiao, K. M., S. L. McMahon, and P. J. Farnham.** 1994. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes Dev. **8:**1526–1537.
19. **Huang, M.-M., and P. Hearing.** 1989. The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. Genes Dev. **3:**1699–1710.
20. **Jakel, S., and D. Gorlich.** 1998. Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J. **17:**4491–4502.
21. **Johnson, D. G., K. Ohtani, and J. R. Nevins.** 1994. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle expression. Genes Dev. **8:**1514–1525.
22. **Johnson, D. G., J. K. Schwartz, W. D. Cress, and J. R. Nevins.** 1993. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature **365:**349–352.
23. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A trans-activation. Cell **45:**219–228.
24. **Krek, W., M. E. Ewen, S. Shirodkar, Z. Arany, W. G. Kaelin, Jr., and D. M.**

Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell **78:** 161–172.

25. **Lang, S., S. McMahon, M. Cole, and P. Hearing.** 2001. E2F transcriptional activation requires TRRAP and GCN5 cofactors. J. Biol. Chem. **276:**32627–32634.
26. **La Thangue, N. B.** 2003. The yin and yang of E2F-1: balancing life and death. Nat. Cell Biol. **5:**587–589.
27. **Magae, J., C. L. Wu, S. Illenye, E. Harlow, and N. H. Heintz.** 1996. Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. J. Cell Sci. **109:**1717–1726.
28. **Marti, A., C. Wirbelauer, M. Scheffner, and W. Krek.** 1999. Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat. Cell Biol. **1:**14–19.
29. **Martinez-Balbas, M. A., U. M. Bauer, S. J. Nielsen, A. Brehm, and T. Kouzarides.** 1998. Regulation of E2F1 activity by acetylation. EMBO J. **19:** 662–671.
30. **Marzio, G., C. Wagener, M. I. Gutierrez, P. Cartwright, K. Helin, and M. Giacca.** 2000. E2F family members are differentially regulated by reversible acetylation. J. Biol. Chem. **275:**10887–10892.
31. **Muller, H., and K. Helin.** 2000. The E2F transcription factors: key regulators of cell proliferation. Biochim. Biophys. Acta **1470:**M1–M12.
32. **Muller, H., M. C. Moroni, E. Vigo, B. O. Petersen, J. Bartek, and K. Helin.** 1997. Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol. Cell. Biol. **17:**5508–5520.
33. **Neill, S. D., C. Hemstrom, A. Virtanen, and J. R. Nevins.** 1990. An adenovirus E4 gene product trans-activates E2 transcription and stimulates stable E2F binding through a direct association with E2F. Proc. Natl. Acad. Sci. USA **87:**2008–2012.
34. **Nevels, M., S. Rubenwolf, T. Spruss, H. Wolf, and T. Dobner.** 2000. Two distinct activities contribute to the oncogenic potential of the adenovirus type 5 E4orf6 protein. J. Virol. **74:**5168–5181.
35. **Obert, S., R. J. O'Connor, S. Schmid, and P. Hearing.** 1994. The adenovirus E4-6/7 protein transctivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. Mol. Cell. Biol. **14:**1333–1346.
36. **O'Connor, R. J., and P. Hearing.** 1991. The C-terminal 70 amino acids of the adenovirus E4-ORF6/7 protein are essential and sufficient for E2F complex formation. Nucleic Acids Res. **19:**6579–6586.
37. **O'Connor, R. J., and P. Hearing.** 2000. The E4-6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection. J. Virol. **74:**5819–5824.
38. **O'Connor, R. J., and P. Hearing.** 1994. Mutually exclusive interaction of the adenovirus E4-6/7 protein and the retinoblastoma gene product with internal domains of E2F-1 and DP-1. J. Virol. **68:**6848–6862.
39. **O'Connor, R. J., J. E. Schaley, G. Feeney, and P. Hearing.** 2001. The p107 tumor suppressor induces stable E2F DNA binding to repress target promoters. Oncogene **20:**1882–1891.
40. **Orlando, J. S., and D. A. Ornelles.** 1999. An arginine-faced amphipathic alpha helix is required for adenovirus type 5 E4orf6 protein function. J. Virol. **73:**4600–4610.
41. **Orlando, J. S., and D. A. Ornelles.** 2002. E4orf6 variants with separate abilities to augment adenovirus replication and direct nuclear localization of the E1B 55-kilodalton protein. J. Virol. **76:**1475–1487.
42. **Palmeri, D., and M. H. Malim.** 1999. Importin β can mediate the nuclear import of an arginine-rich nuclear localization signal in the absence of importin α. Mol. Cell. Biol. **19:**1218–1225.
43. **Pierce, A., R. Schneider-Broussard, J. Philhower, and D. Johnson.** 1998. Differential activities of E2F family members: unique functions in regulating transcription. Mol. Carcinog. **22:**190–198.
44. **Pierce, A. M., S. M. Fisher, C. J. Conti, and D. G. Johnson.** 1998. Deregulated expression of E2F1 induces hyperplasia and cooperates with ras in skin tumor development. Oncogene **16:**1267–1276.
45. **Pierce, A. M., I. B. Gimenez-Conti, R. Schneider-Broussard, L. A. Martinez, C. J. Conti, and D. G. Johnson.** 1998. Increased E2F1 activity induces skin tumors in mice heterozygous and nullizygous for p53. Proc. Natl. Acad. Sci. USA **95:**8858–8863.
46. **Qin, X. Q., D. M. Livingston, W. G. Kaelin, Jr., and P. D. Adams.** 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. USA **91:**10918–10922.
47. **Schaley, J., R. J. O'Connor, L. J. Taylor, D. Bar-Sagi, and P. Hearing.** 2000. Induction of the cellular E2F-1 promoter by the adenovirus E4-6/7 protein. J. Virol. **74:**2084–2093.
48. **Sears, R., K. Ohtani, and J. R. Nevins.** 1997. Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. Mol. Cell. Biol. **17:**5227–5235.
49. **Shan, B., and W. H. Lee.** 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. **14:**8166–8173.
50. **Singh, P., S. H. Wong, and W. Hong.** 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. EMBO J. **13:**3329–3338.
51. **Stevaux, O., and N. J. Dyson.** 2002. A revised picture of the E2F transcriptional network and RB function. Curr. Opin. Cell Biol. **14:**684–691.
52. **Stevens, C., and N. B. La Thangue.** 2003. E2F and cell cycle control: a double-edged sword. Arch. Biochem. Biophys. **412:**157–169.
53. **Takahashi, Y., J. B. Rayman, and B. D. Dynlacht.** 2000. Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev. **14:**804–816.
54. **Truant, R., and B. R. Cullen.** 1999. The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin β-dependent nuclear localization signals. Mol. Cell. Biol. **19:**1210–1217.
55. **Verona, R., K. Moberg, S. Estes, M. Starz, J. P. Vernon, and J. A. Lees.** 1997. E2F activity is regulated by cell cycle-dependent changes in subcellular localization. Mol. Cell. Biol. **17:**7268–7282.
56. **Xiao, W., K. H. Warrington, Jr., P. Hearing, J. Hughes, and N. Muzyczka.** 2002. Adenovirus-facilitated nuclear translocation of adeno-associated virus type 2. J. Virol. **76:**11505–11517.
57. **Xu, G., D. M. Livingston, and W. Krek.** 1995. Multiple members of the E2F transcription factor family are the products of oncogenes. Proc. Natl. Acad. Sci. USA **92:**1357–1361.
58. **Xu, M., K.-A. Sheppard, C.-Y. Peng, A. S. Yee, and H. Piwnica-Worms.** 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Cell. Biol. **14:**8420–8431.
59. **Yamasaki, L., T. Jacks, R. Bronson, E. Goillot, E. Harlow, and N. J. Dyson.** 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell **85:** 537–548.
60. **Yeh, C. T., Y. F. Liaw, and J. H. Ou.** 1990. The arginine-rich domain of hepatitis B virus precore and core proteins contains a signal for nuclear transport. J. Virol. **64:**6141–6147.